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# Visualization of Chromosomal Domains with Boundary Element-Associated Factor BEAF-32

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## Summary

**We have purified two proteins from Drosophila that bind to the scs′ boundary element of the *hsp70* domain at locus 87A7. Their palindromic binding sites (CGATA-TATCG) symmetrically abut the previously mapped hypersensitive site of scs′. We have cloned a cDNA for one of these proteins, BEAF-32 (boundary element-associated factor of 32 kDa). It encodes a novel protein that is bound to scs′ but not scs in vivo. Immunostaining localizes BEAF to hundreds of interbands and many puff boundaries on polytene chromosomes, suggesting that a chromosomal domain consists of a band (or puff) and part of the flanking interbands. Enhancer blocking assays implicate the palindromic binding site in boundary function. The lack of enhancer blocking in transiently transfected cells suggests an involvement of chromatin, nuclear structure, or both in boundary function.**

## Introduction

Current thinking about the functional organization of the eukaryotic genome is dominated by the notion that chromatin is partitioned into largely independent units called domains. Experimental evidence for domains is based on the observation that placing certain large-sized DNA fragments on both sides of reporter genes suppresses the position-dependent expression levels normally observed after integration into the genome (see, e.g., Grosveld et al., 1987). Apparently, these flanking elements somehow neutralize the repressive effects of the local chromatin structure on reporter gene expression, while also inhibiting heterologous enhancer interactions with the reporter gene promoter that are most dramatically observed in enhancer trap experiments. Although the mechanisms involved are not understood, these elements are thought to bring about domain independence by long-range and local alterations of chromatin structure, possibly involving nuclear compartmentalization. It is of importance to identify the various elements necessary to define a domain completely and to understand their mechanisms of action.

In this report, we are concerned with characterizing a class of elements thought to define the periphery of a domain, called the boundary element (BE). Important progress toward this goal was made by Schedl and collaborators through the discovery and characterization of two special chromatin structures (scs and scs′) defined by nuclease-hypersensitive sites bracketing a resistant region (Udvardy et al., 1985; reviewed by Vazquez et al., 1993). On the basis of the localization of these putative BEs to the puff boundaries of the Drosophila melanogaster *hsp70* domain at locus 87A7, they could play an important role in limiting the spread of open and closed chromatin states. As expected for a bona fide BE, they can insulate a reporter gene at most sites of insertion (not in heterochromatin) from the effects of the local chromatin environment in transgenic Drosophila, dramatically reducing position-dependent variability of reporter gene expression (Kellum and Schedl, 1991), as well as blocking the action of an enhancer when positioned between the enhancer and promoter (Kellum and Schedl, 1992; Vazquez and Schedl, 1994). In addition, these BEs appear to have no enhancer activity on their own.

Enhancer-blocking activity has also been described for the 5′-most constitutive nuclease-hypersensitive site (called 5′HS4) upstream of the locus control region (LCR) of the chicken β-globin domain (Chung et al., 1993). 5′HS4 lacks enhancer activity, can insulate a reporter gene from the activating effects of a nearby LCR in human cells, and, remarkably, can confer position-independent expression to the *white* minigene in transgenic Drosophila. LCRs (reviewed by Engel, 1993; Dillon and Grosveld, 1993), in contrast, are tissue-specific putative domain elements capable of conferring high level reporter gene expression; they contain a high concentration of binding sites for general and tissue-specific transcriptional regulatory proteins and are associated with multiple tissue-specific hypersensitive sites and the decondensation of higher order chromatin structures over the domains they regulate. The insulating effect of the constitutively nuclease-hypersensitive 5′HS4 element appears to be separate and distinct from the other functions of LCRs associated with the tissue-specific hypersensitive sites. If 5′HS4 is typical, perhaps LCRs are generally associated with a 5′ BE.

Similar enhancer blocking and position-independent expression is conferred by reiterated binding sites of the suppressor of Hairy wing (su(Hw)) protein, such as those found in the *gypsy* retrotransposon (Geyer and Corces, 1992; Roseman et al., 1993). The efficiency of enhancer blocking is related to the number of reiterated su(Hw)-binding sites. Interestingly, mutation of a second locus (*mod(mdg4)*) eliminates the directionality of the blocking effect (reviewed by Lewin, 1994). However, this case is so far unique in that enhancer blocking by su(Hw) has been shown to occur in transient assays (Holdridge and Dorsett, 1991), suggesting that higher order chromatin organization is not involved.

We concentrated our efforts on characterizing proteins that bind the scs′ boundary element of the 87A7 locus. Purification of a protein doublet (BEAF, for boundary element–associated factor) from Drosophila that binds with high affinity to a palindromic sequence found in scs′ led to cDNA cloning and characterization of a novel protein

*These authors contributed equally to this work.

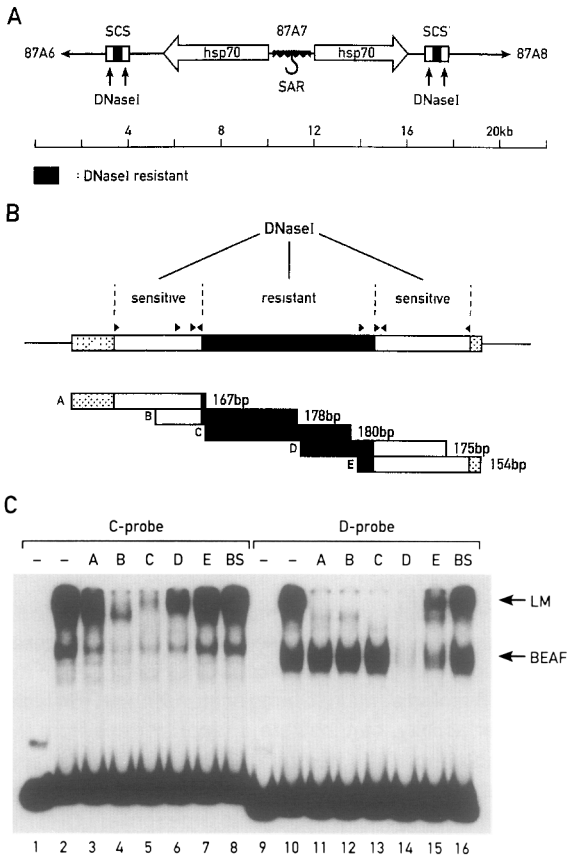


Figure 1. Identification of scs'-Binding Proteins

(A) Map of the 87A7 heat shock locus, showing the SAR, the two divergently transcribed *hsp70* genes, and the scs and scs' elements. Also indicated are the DNase I–hypersensitive (arrows) and DNase I–resistant (closed box) regions of scs and scs' (not drawn to scale).
(B) Map relating the location of the DNase I–hypersensitive and DNase I–resistant regions in the scs' core to the five subfragments used in protein binding assays (shown underneath). The line represents cloning vector sequences. Also indicated are the positions and orientations of the CGATA sequence motif (arrowheads; see text for details).
(C) Gel shift analysis with 2 μg of KC nuclear extract (no protein in lanes 1 and 9), 5 μg of poly(dI–dC), 1 fmol of labeled C (lanes 1–8) or D (lanes 9–16) probe, and a 150-fold molar excess of the indicated competitor DNAs. BS is a 170 bp fragment from pBluescript KS. The LM and BEAF complexes are indicated.

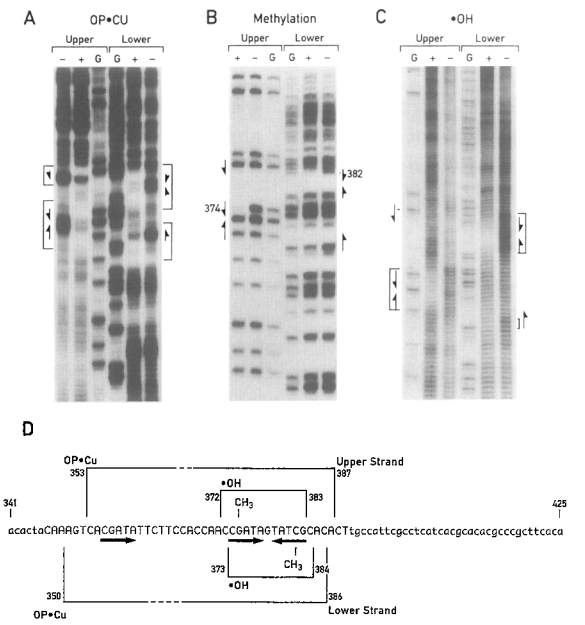


Figure 2. Footprint and Interference Analysis of BEAF Binding to the scs' D Subfragment

(A) Footprint after OP-Cu treatment of bound and free DNA. PC-purified BEAF was allowed to bind to the D probe end-labeled on either the upper or lower strand, followed by separation of free and bound DNA on a native 4% polyacrylamide gel. The gel was treated with OP-Cu, and the free (minus lanes) and bound (plus lanes) DNA was isolated and analyzed on a 6% sequencing gel together with G reaction size standards of the same DNA. Arrows indicate the locations of CGATA motifs.
(B) Methylation interference analysis of binding. Similar to (A), except instead of OP-Cu treatment, the DNA was methylated by DMS prior to protein binding, followed by cleavage of the methylated DNA with piperidine after isolation of the free (minus lanes) and bound (plus lanes) DNA. Also, the protein used had been further purified by two passages over a DNA affinity column made with the BTS oligonucleotide, which encodes 44 bp of scs' sequences encompassing the OP-Cu footprint (see [D]).
(C) Missing nucleoside analysis of binding. Similar to (A), except instead of OP-Cu treatment, nucleosides were removed by treatment with hydroxyl radical prior to protein binding, and the isolated free (minus lanes) and bound (plus lanes) DNA was analyzed on an 8% sequencing gel. The protein used was affinity purified as in (B).
(D) Summary of the footprinting data. Only the sequence of the upper strand is shown, together with the nucleotide coordinates. The nucleotides important for binding as defined by the methylation interference ($CH_3$) and missing nucleoside (OH) analyses, as well as the region protected from reaction with OP-Cu, are shown, together with the CGATA sequence motifs (arrows). The BTS oligonucleotide used to affinity-purify BEAF is indicated by capital letters.

called BEAF-32 (BEAF of 32 kDa molecular mass). We identify BEAF together with the palindromic binding site as components implicated in scs' boundary function. Importantly, BEAF localizes by immunofluorescence to numerous interbands of polytene chromosomes, suggesting a link between chromosomal domains and cytological markers. The body of a chromosomal domain is morphologically defined on polytene chromosomes by a band, and its boundary by part of the flanking interbands.

## Results

### Identification of Nuclear Proteins Interacting with the scs' Core

We focused our experimental attention on scs', the smaller of the two boundary elements associated with the *hsp70* genes at locus 87A7 (Figure 1A). The scs' core (515 bp) was subcloned into five overlapping fragments of less than 200 bp each (Figure 1B) to facilitate gel shift and footprinting experiments. Two major DNA-binding activities were detected by gel shift with crude Drosophila KC nuclear extracts, a sequence-specific binding activity with a high affinity for the D subfragment, called the BEAF complex, and a low mobility complex (LM; Figure 1C). The LM complex appears to interact less specifically, being observed with all scs' subfragments. However, it is not observed with the Bluescript control fragment. Of possible

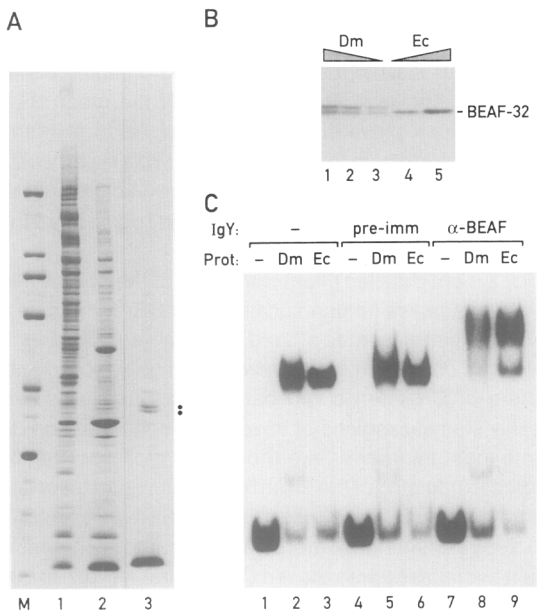


Figure 3. *BEAF-32* Encodes One Protein of the BEAF Doublet

(A) Purification of the BEAF doublet from KC nuclei, visualized by Coomassie staining after 7.5%–15.0% SDS–PAGE. Lane 1, KC nuclear extract. Lane 2, protein profile of the fraction containing BEAF activity after one round of BTS DNA affinity purification. Lane 3, protein profile of the fraction containing BEAF activity after a second round of BTS DNA affinity purification. Lane M, molecular mass markers of, from the top, 205, 116, 97, 68, 45, and 29 kDa. The apparent molecular masses of the purified proteins are 38 kDa and 40 kDa. In this purification, the PC column was omitted.

(B) Western blot analysis of various concentrations of isolated KC nuclei and BEAF-32 purified from E. coli, after separation by 7.5%–15.0% SDS–PAGE. The bacterially expressed protein migrates at the same position as the lower BEAF protein. The rabbit antibody raised against bacterial BEAF-32 was used, implying that the two Drosophila proteins are related. Nuclei (0.5, 0.25, and 0.125 $OD_{260}$ units) were loaded onto lanes 1, 2, and 3, respectively; 40 ng and 80 ng of purified BEAF-32 were loaded onto lanes 4 and 5, respectively. Quantitation of BEAF from blots such as this gives a value of approximately one doublet per 10 kb of nuclear chromatin.

(C) BEAF (Dm lanes) and bacterially expressed BEAF-32 (Ec lanes) make complexes with the D subfragment that have similar gel shifts (lanes 1–3). Addition of affinity-purified chicken antibody raised against the upper protein of the BEAF doublet leads to a similar supershift of both the Drosophila and bacterially expressed BEAF-32 protein–DNA complexes (lanes 7–9), whereas preimmune immunoglobulins from chicken egg yolks (IgYs) have no effect (lanes 4–6).

interest is the hierarchy of binding affinities of the LM complex for the various scs′ subfragments, as indicated by competiton experiments (Figure 1C; data not shown). In contrast, the BEAF complex is specific for the D probe; addition of a 150-fold molar excess of the unlabeled D (not A, B, C, or Bluescript), and to some extent the partially overlapping E fragment, results in dissociation of the radiolabeled complex (Figure 1C).

Fractionation of the crude nuclear extracts by phosphocellulose P11 (PC) chromatography separates the LM complex (which flows through at 250 mM NaCl) from the BEAF activity (elution at 370–420 mM NaCl). Utilizing this separation, the BEAF DNA target sequences were identified by using three different footprint techniques involving isolation of the free and bound DNA after gel shift analysis. The orthophenanthroline–copper (OP-Cu) footprint identifies two closely spaced regions on both strands (Figure 2A). This sequence contains an inverted repeat of the pentanucleotide sequence CGATA separated by one base pair, and an additional copy of the CGATA sequence. These motifs are represented by arrows in all panels of Figure 2. Binding to the DNA was impaired when nucleosides were removed from the inverted repeat by hydroxyl radical treatment (Figure 2C) or when either inner G was methylated in the major groove by dimethyl sulfate (DMS) (Figure 2B), confirming the importance of the inverted repeat. Neither methylation of the outer Gs nor methylation in the minor groove of the As affected protein binding. Similarly, alteration of the single CGATA element had very little effect on protein binding.

The footprinting data, summarized in Figure 2D, identify the inverted repeat as the important binding site. A much weaker interaction occurs at the single CGATA motif. An examination of the entire scs′ core sequence revealed five additional copies of the CGATA motif, two of which are in an inverted orientation separated by 3 bp and are present in the B subfragment (see Figure 1B). This fragment is also bound by BEAF, but with a reduced affinity (data not shown). It is of interest to note the symmetric distribution of the CGATA motifs with respect to the nuclease-hypersensitive and nuclease-resistant regions (Udvardy et al., 1985; Farkas and Udvardy, 1992), with single copies being found at the outer ends of the hypersensitive regions and palindromes with associated single copies bracketing the central resistant region (see Figure 1B).

### Purification and Isolation of the *BEAF-32* cDNA Clone

The BEAF activity was purified by DNA affinity chromatography using a concatenated 48 bp oligonucleotide encompassing the OP-Cu footprint. This sequence is indicated by capital letters in Figure 2D and for convenience referred to as BTS (for BEAF target sequence). Nuclear extracts prepared from KC cells were fractionated on a PC column, and the BEAF activity was further purified by two passages over a BTS affinity column in the presence of poly(dI–dC) (Kadonaga, 1991). A major protein doublet with apparent molecular masses of 38 kDa and 40 kDa (Figure 3A) comprises the only detectable proteins whose concentration profile matched the BEAF activity profile in the various column fractions. Cyanogen bromide peptide mapping (data not shown), peptide sequencing (indicated in Figure 4), and immunoreactivity (discussed below) indicate that the two proteins are either closely related, posttranslational variants, or both.

We cloned a cDNA encoding one of the proteins (BEAF-32) of this DNA-binding activity after obtaining sequences of internal tryptic peptides derived from the separated proteins of the doublet. A sequence from the upper protein was used to design an oligonucleotide of low degeneracy for use in a rapid amplification of cDNA ends (RACE) protocol (Frohman et al., 1988). The resulting 800 bp fragment was used as a probe to screen a 4–8 hr embryo cDNA library (Brown and Kafatos, 1988), and a single cDNA

```
  1  MHVEKKSELRSLLKSGDKRCKLVEPRNTKSCVWRFFNLVQCDDHIEPYAC
 51  CKTCGDLLSYSGKTGTGSLLRHRCLHSSSSNDKTVRITKAKTLREPLRVA
101  KPKIESNVANYLGEAGALPQKWEEYEQNDETSEDIKDIIYSEDPLCYSPI
151  HVMDDEGLDQPEKQVTVLTHSTSPAGGSSRPIGVGSGVQATVVASTSSSS
201  SSAKQLKNNLETSIERLTAVSEQLSYIIQQNHEELTKDDDYYFALSLVPA
                ^^^^^^^^^^^^^^^
251  MRHLSLSRKMYVRSKIQDILFKESEDSTLAKDE*
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Figure 4. Predicted Amino Acid Sequence of the *BEAF-32* cDNA

Amino acids are numbered on the left from the first methionine, with the termination codon indicated by an asterisk. Lines over the amino acid sequence represent tryptic peptide sequences obtained from the upper protein of the BEAF doublet, and lines under the amino acid sequence represent tryptic peptide sequences obtained from the lower protein. Also indicated under the sequence is an atypical leucine zipper sequence (carets). Potential phosphorylation sites for casein kinase II are at T53, S79, T92, T212, and T236; for protein kinase C, at S61, T84, T92, S178, S202, and S257; and for tyrosine kinase, at Y60. Differential phosphorylation of these sites could account for the heterogeneous charge content observed by two-dimensional gel electrophoresis (data not shown). Another notable feature of BEAF-32 is its serine richness (12.5%) with short clusters around amino acids 77 and 195.

clone was isolated. This cDNA contains an open reading frame encoding a protein of 283 amino acids with a calculated molecular mass of 31.6 kDa (hence, BEAF-32; Figure 4). This reading frame encodes several of the tryptic peptide sequences previously obtained from both the lower and upper proteins of the doublet (indicated in Figure 4).

BEAF-32 appears to be a novel protein, lacking obvious similarities to known proteins or structural motifs. In situ hybridization with the cDNA probe mapped the *BEAF-32* gene to 51D1 of the polytene chromosome arm 2R, and Southern blot analysis indicated a single-copy gene for BEAF-32 (data not shown).

We next compared biochemical properties of BEAF-32 with those of the BEAF doublet. As evident from the Western blot in Figure 3B, the BEAF doublet is recognized by an affinity-purified rabbit antibody raised against bacterially produced BEAF-32, and BEAF-32 has the same mobility as the lower band of the BEAF doublet during SDS–polyacrylamide gel electrophoresis (SDS–PAGE). A chicken antibody directed against the upper band of the BEAF doublet also recognizes both Drosophila proteins as well as bacterially expressed BEAF-32. In addition, purified BEAF-32 shifts the D probe in a similar manner as the Drosophila BEAF activity, and both give a similar supershift in the presence of the chicken antibody mentioned above (see Figure 3C). Combined with the peptide sequencing data, these data identify BEAF-32 as one component of the BEAF-binding activity. Yet experiments to be published elsewhere demonstrate that BEAF-32 does not fully reconstitute the BEAF DNA-binding activity (see Discussion).

### BEAF Immunolocalizes to Interbands and Puff Boundaries on Polytene Chromosomes

Immunological work using Drosophila adults, embryos, and cell lines with the affinity-purified rabbit antibodies against BEAF-32 identifies the BEAF doublet as two ubiquitous nuclear proteins also present on mitotic chromosomes (data not shown). Interestingly, in situ BEAF staining does not simply follow total DNA but is restricted to discrete subnuclear regions and is excluded from the nucleolus. This is illustrated by the nuclear staining pattern of D1 cells shown in Figure 5F. Clearly the green BEAF signal (regions of overlap appear yellow) yields an internal pattern different from the red DNA stain. This is particularly evident at the nuclear periphery, where exclusion of the protein stain can be noted in DNA-dense regions that may represent heterochromatin.

For a more detailed characterization of their distribution on chromatin, we immunolocalized the BEAF proteins on polytene chromosomes of third instar larvae. Staining the DNA with propidium iodide reveals a highly reproducible pattern of DNA-dense red bands separated by darker, weakly staining regions of lower DNA concentration, the interbands. Interbands are thought to represent regions of partial unfolding of the 30 nm chromatin fiber associated with the potential for transcriptional activity (Rykowski et al., 1988). Staining by the BEAF-32 antibody (green, or, when overlapping with the red DNA signal, yellow) is quite striking and restricted to many inter- or near interband regions (Figures 5A and 5B). The chromocenter, which consists of the heterochromatic, satellite DNA–rich centromeres of polytene chromosomes, does not immunostain (Figure 5A, arrowhead). The interband-type immunostaining is readily apparent at higher magnification, as shown in Figure 5B. Inclusion of the mirror image showing only the DNA staining facilitates localization of the BEAF signal with respect to the band/interband pattern. Comparison of the two images localizes the BEAF signal generally to interbands, as illustrated by a few vertical connecting lines. Interestingly, staining by the BEAF antibody is restricted to perhaps 50% of inter- or near interband regions and appears quite clustered in certain chromosomal regions.

The scs and scs′ elements were originally identified as specialized chromatin structures that map by in situ hybridization to the borders of the heat shock puff at the 87A7 locus (Udvardy et al., 1985). Consequently, we would expect the BEAF-32 antibody to stain at least one edge of this puff. Furthermore, if puff borders represent boundary elements and if BEAF is a general factor involved in the formation of other boundary elements, we would expect to observe a similar staining of other puff borders. Indeed, this is generally observed. Figure 5D shows strong staining at the distal, scs′-containing side (arrowhead) of the 87A7 heat shock puff, whereas the proximal, scs-containing side shows only extremely weak or no staining. This is consistent with our failure to detect BEAF binding to the scs element by gel shift assay. BEAF localizes to the proximal but not the distal edge of the adjacent heat shock puff at 87C. Although we have not completed a systematic survey of puff staining, we have observed that BEAF localizes to the base or edge of many puffs and often is found at only one edge of a given puff. By way of example, Figure 5C shows the major developmental puffs at 75B and 74EF sometimes called the Chinese lantern. The former puff appears bracketed on one and the latter on either side by BEAF. The unidentified puff in Figure 5E provides an example of an atypical puff that is subdivided by several

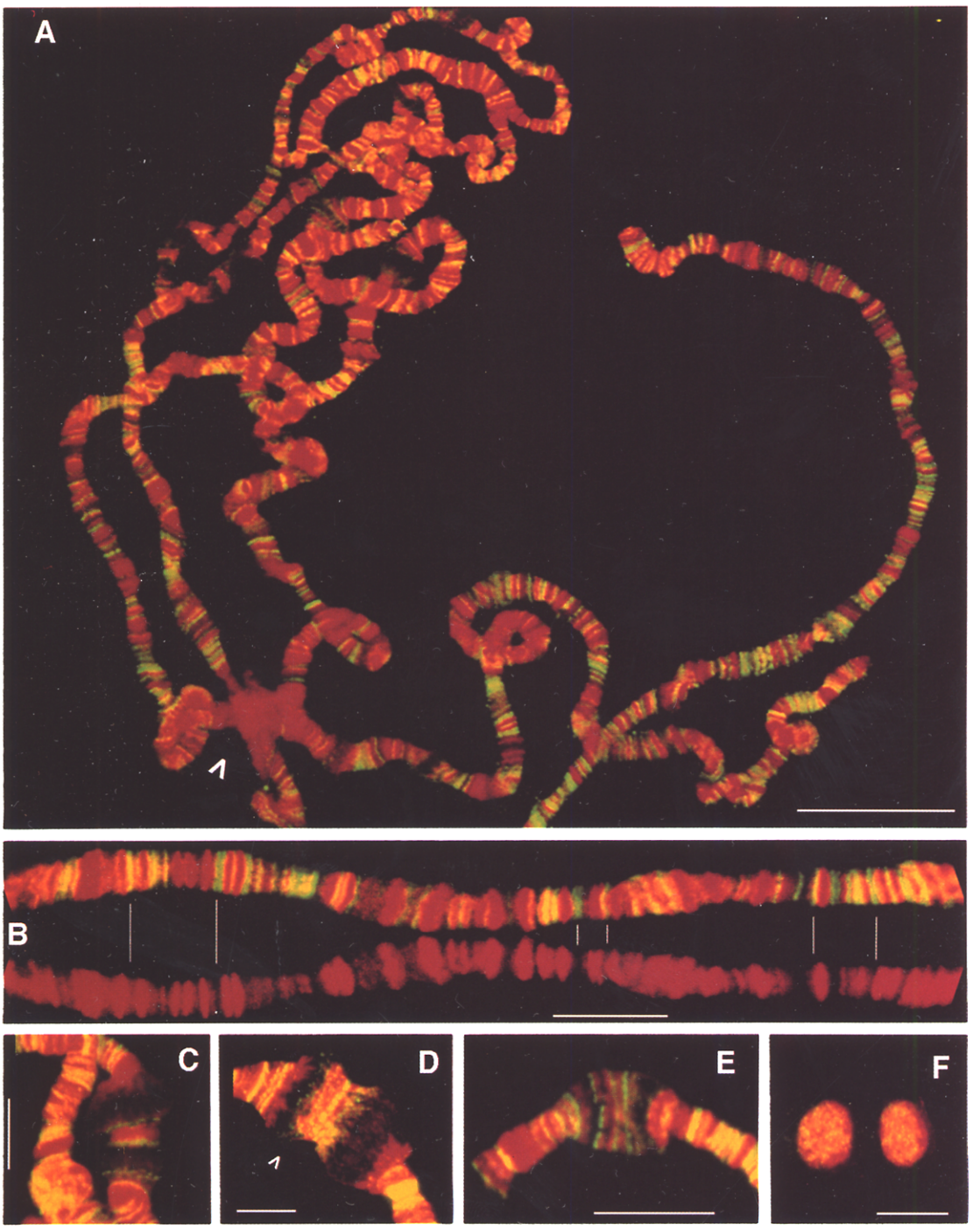



Figure 5. BEAF Immunolocalizes to Interbands and Puff Borders of Polytene Chromosomes

Polytene chromosomes were stained for DNA with propidium iodide (red) to highlight the banding pattern: DNA-dense bands appear bright red, and the less dense interbands are darker. The BEAF-32-directed antibody signal is green (regions of overlap with DNA appear yellow), arising from the FITC-conjugated secondary antibody. Unless otherwise noted, all images have the BEAF-32 and DNA signals merged.

(A) Survey of an entire polytene chromosome spread. Note the numerous discrete BEAF signals throughout the chromosomes, and exclusion of BEAF from the heterochromatic chromocenter (indicated by an arrowhead).

(B) Mirror images of an enlarged chromosomal section. Only the red DNA signal is displayed in the lower image to highlight the band/interband structure. The mirror symmetry facilitates comparision of the top and bottom images, demonstrating the interband-type staining pattern of BEAF-32. A few examples are indicated by vertical lines.

(C–E) Examples of the puff boundary localization of BEAF are shown in (C) (developmental puffs 74EF and 75B), (D) (heat shock puffs at 87A7, with the scs′ side indicated by an arrowhead, and 87C), and (E) (an unidentified puff).

(F) Nuclear staining of D1 cells.

Scale bar in (A), 25 μm; in other panels, 10 μm.

DNA-dense red striations, with BEAF localizing to several edges. These immunolocalization studies encourage the notion that BEAF proteins are involved in the longitudinal partitioning of chromosomes into functional units, and they also suggest the possibility that many domains are bracketed by different classes of boundary elements.

### BEAF Is Associated with the scs′ Element In Vivo

To confirm the relevance of the in vitro interaction and immunolocalization studies, the in vivo interaction of BEAF with chromatin was analyzed. We fixed the distribution of BEAF on chromatin in intact cells by cross-linking with formaldehyde (Solomon et al., 1988). Formaldehyde-fixed KC cells were lysed, and the cross-linked, fragmented chromatin was isolated on CsCl gradients prior to immunoprecipitation of 30 μg aliquots with or without affinity-purified anti-BEAF-32 antibodies (Orlando and Paro, 1993). About 0.01% of the input DNA–protein complex was recovered both in the presence and absence of the specific antibody. This DNA, after decross-linking, was amplified by ligation-mediated polymerase chain reaction (PCR) (Mueller and Wold, 1989) to obtain enough DNA for further analysis.

The amplified DNA was slot-blotted and hybridized with different probes. To obtain a semiquantitative estimate, we loaded about 50 ng of the immunoprecipitated DNA into the top slot and increasing amounts (0.1–10.0 μg) of total KC DNA into the slots below. As shown in Figure 6, the scs′ sequence is significantly enriched in the immunoprecipitated DNA. We estimate the enrichment of scs′ sequences to be 1500-fold relative to the actin 5C control DNA, or 500-fold relative to total genomic DNA. Although the scs element appears to lack *BEAF* sites, we observe a slight enrichment (3- to 5-fold relative to actin 5C) of the scs signal in the immunoprecipitated DNA. This observation could possibly reflect an interaction (perhaps via chromatin looping) between the scs and scs′ boundary elements. As a further control, no enrichment of scs′ sequences was detectable in the DNA precipitated in the absence of antibody (data not shown).

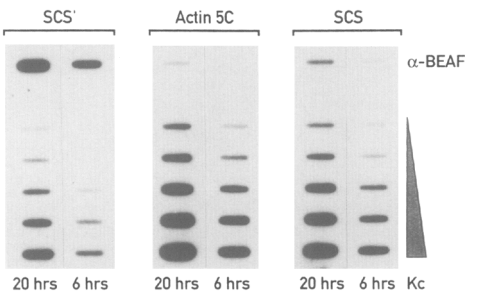



Figure 6. BEAF Binds to scs′ In Vivo

Cross-linked protein–DNA complexes were immunoprecipitated with affinity-purified anti-BEAF-32 antibody and protein A–agarose after isolation from formaldehyde-treated KC cells by isopycnic CsCl centrifugation. After removal of protein, the immunoprecipitated DNA was amplified by LM–PCR and 50 ng applied to a nylon filter by slot blot, together with 0.1, 0.3, 1.0, 3.0, and 10.0 μg of total genomic DNA as concentration standards. Filters were hybridized against an scs′, actin 5C, or scs probe as indicated. Two autoradiographic exposures are shown to aid quantitation.

### BEAF Contributes to the Boundary Function of the scs′ Element

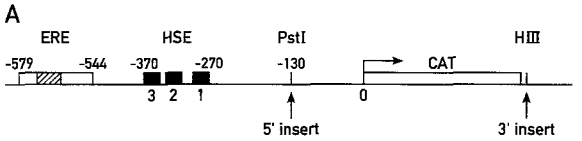
Kellum and Schedl (1992) have demonstrated that the scs′ and scs elements, when inserted between the *yp-1* enhancer and *hsp70* promoter in transgenic flies, can block the *yp-1* enhancer. To assess the role of the BEAF target sequences in scs′ function, we set up a blocking assay using a Drosophila cell line (D1) suitable for the selection of stable transformants (Dübendorfer, 1986; Rio and Rubin, 1985). A well-characterized Drosophila *hsp27* promoter CAT construct served as the reporter (Riddihough and Pelham, 1986). This promoter contains ecdysone response elements (ERE) and heat shock regulatory elements (HSE) as shown in Figure 7A. Candidate boundary elements were inserted into the PstI site at −129, and in the case of the double constructs also into a HindIII site 3′ of the gene (Figure 7A). The level of CAT activity was analyzed in stably transformed cells following heat shock or ecdysone treatment, and in a transient assay after ecdysone treatment. Note that the stably transformed cells were assayed by pooling several hundred individual clones and, consequently, are reflective of many genomic insertion sites. The data summarized in Figure 7B are expressed as repression relative to the level of expression of the construct containing the 170 bp Bluescript fragment (BS, construct 2), so that higher values indicate stronger enhancer blocking.

These data show that elements containing the palindromic BEAF-binding site block the ecdysone response in stably, but not transiently, transfected cells. Repression was greatest for the 515 bp scs′ core (constructs 3 and 4), but was also significant for the 175 bp D (construct 5) and the 7-mer repeat of the 48 bp BTS oligonucleotide (constructs 7 and 8). Repression was greater when a second copy of the test element was included downstream of the CAT gene, an effect thought to result from insulation against 3′ enhancer activity arising from tandemly inserted copies that are often generated by integration events.

Repression by the D fragment is reduced about 2.3-fold upon mutation of the palindromic CGATA sequences to C*TCGA* (mD, construct 6), while introducing this mutation into all CGATA sequences in the 7-mer repeat of BTS (m2-BTS) reduced the level of repression nearly 3-fold (constructs 9 and 10). Both mutations abolished BEAF binding in vitro (data not shown). We consider this reproducible but partial loss of repression of significance. While this suggests that other proteins (perhaps those involved in forming the low mobility complex; see Figure 1C) and sequences are involved, these data nevertheless implicate the CGATA repeats in boundary function and, by extension, the BEAF protein doublet.

In contrast with the effects on ecdysone induction, all enhancer-blocking constructs had only a 3- to 5-fold repressive effect on CAT activation by heat shock. The presence of mutations did not affect this. One possible explanation is that the close proximity of the HSE destabilizes the boundary elements.

A




B

| | Insert | | Transient | Stable | |
|---|---|---|---|---|---|
| | 5' | 3' | ecd | ecd | hs |
| 1 | – | – | 0.5x | 1.7x | 0.4x |
| 2 | BS | – | 1.0x | 1.0x | 1.0x |
| 3 | SCS' | – | 1.8x | 25.0x | 3.5x |
| 4 | SCS' | SCS' | 0.8x | 38.0x | 5.4x |
| 5 | D | – | 1.8x | 6.9x | 3.3x |
| 6 | mD | – | 1.7x | 3.0x | 2.1x |
| 7 | 7mer | – | 1.2x | 11x | 2.9x |
| 8 | 7mer | 7mer | nd | 14.3x | 4.4x |
| 9 | m2 7mer | – | 1.2x | 4.2x | 3.2x |
| 10 | m2 7mer | m2 7mer | nd | 4.8x | 2.4x |

Figure 7. The BEAF-Binding Site Contributes to Boundary Function In Vivo

(A) Schematic representation of the *hsp27* promoter–CAT reporter gene construct used in transient and stable transfections of the Drosophila D1 cell line. A 1200 bp *hsp27* promoter fragment was used. The positions of the ERE, HSEs, and 5′ and 3′ insertion sites for boundary elements are shown.

(B) Fold repression of CAT activities, normalized to the 170 bp pBluescript KS fragment control element, assayed in cell extracts prepared from transient or stable transfectants after ecdysone or heat shock treatment as indicated. The 7-mer and m2 7-mer consist of seven head-to-tail copies of the BTS and m2-BTS oligonucleotides respectively, joined by BamHI and BglII sticky ends; other DNA fragments tested for boundary activity are described in the text.

## Discussion

### The BEAF Activity, the CGATA Elements, and the Specialized Chromatin Structure at scs′

We purified the BEAF DNA-binding activity from KC cell nuclear extracts on the basis of its specificity for the D subfragment of the scs′ boundary element from the 87A7 *hsp70* domain. BEAF specifically binds the CGATA palindrome containing a single nucleotide spacer as well as the single CGATA motif present in the D fragment. The missing nucleoside and methylation interference experiments indicated that only the palindrome is necessary for binding and that major groove interactions are important.

Our data support the notion that BEAF and the CGATA elements are involved in the formation of the special chromatin structure of scs′. The CGATA elements of D are located near the junction of the scs′ nuclease-resistant core and the right hypersensitive region (Farkas and Udvardy, 1992; Figure 1B), and in vitro binding of BEAF to this region induces major DNase I–hypersensitive sites on either side of the CGATA palindrome (C. M. H., K. Z., and U. K. L., unpublished data). We have shown that BEAF interacts with scs′ in vivo, so these sites could be identical with or contribute to the hypersensitive sites observed in chromatin by Udvardy et al. (1985). Moreover, the junction of the resistant core and the left hypersensitive region structurally appears to be a repeat of the right side, containing a CGATA palindrome as well as a single CGATA sequence. This region is on the B fragment, to which BEAF also specifically binds, albeit with a reduced affinity (presumably because the palindrome has a 3 bp, rather than 1 bp, spacer; Figure 1C, lane 12 versus lane 14; data not shown). Perhaps the right and left sides of the scs′ nuclease-resistant core interact cooperatively, conceivably trapping a nucleosome in the roughly 200 bp core. It is further possible that the single CGATA motifs bracketing the hypersensitive regions join in these interactions. It will be of interest to attempt reconstruction of this specialized chromatin structure using histones and BEAF.

### The Morphological Polytene Chromosome Band/Interband Structure Corresponds to a Chromosomal Domain

Immunological techniques revealed a restricted or localized association of BEAF with chromosomes. BEAF was localized in vivo to the scs′ element, but not the actin 5C gene or scs, by immunoprecipitation of formaldehyde cross-linked chromatin. Consistent with the lack of a CGATA palindrome, scs was only slightly enriched in this specific DNA subfraction. This implicates other proteins in scs formation and possibly indicates a weak interaction between scs′ and scs in vivo.

The restricted distribution of BEAF is most dramatically demonstrated by immunofluorescence on polytene chromosomes. The association of BEAF with many interbands and puff edges suggests that it may have quite a general structural/functional role in defining many boundary elements throughout the Drosophila genome, while the one-sided staining suggests that many chromosomal domains have a polar organization (for instance, with an scs′ [BEAF] and an scs side). Identification of scs-binding proteins will help resolve this question.

BEAF localizes differently than other characterized proteins. For example, topoisomerase II mainly stains bands (Heller et al., 1986), and topoisomerase I mainly stains regions of high transcriptional activity (Fleischmann et al., 1984). Although RNA polymerase II localizes to most interbands and puffs, it homogeneously stains puffs and possibly also interbands (Weeks et al., 1993; Jamrich et al., 1977). Another abundant chromosomal protein, GAGA factor, also homogenously stains puffs (Tsukiyama et al., 1994). GAGA also stains many other chromosomal positions, but these were not compared with the polytene DNA banding pattern.

By quantitative immunoblots, we estimate there to be about one molecule of both proteins in the BEAF doublet per 10 kb of DNA in KC cells. This estimate fits well with a putative structural role in chromosome domains. At approximately 22 kb per average band/interband (Ashburner, 1989), there are enough BEAF dimers to interact with every band/interband border. However, if scs′ is typical, then two or even four dimers could bind per element. In this case, there is enough BEAF to interact with about 25% or 50% of band/interband borders (50%–100% of interbands), as observed.

Chromosomal domains can be linked to cytological markers by using these data. The body of a domain generally appears to be represented by a polytene band, and its boundary by part of the flanking interbands. Of course, the BEAF-defined domains may be further subdivided (structurally and functionally).

### BEAF-32

Our data show that the *BEAF-32* cDNA encodes a novel protein that certainly represents one component of the Drosophila BEAF activity. Yet detailed DNA binding and footprinting studies indicate that their DNA binding specificities are not completely identical. Both interact with CGATA motifs in the D fragment, but while the inverted repeat is essential for BEAF binding, BEAF-32 interacts with the single CGATA sequence and then "jumps" over the inverted repeat to interact with sequences from 400 to 417 (C. M. H., K. Z., and U. K. L., unpublished data). BEAF consists of a doublet of related proteins, judging by peptide sequence and immunological data. These proteins could be splicing variants, posttranslational variants, or both, since our genomic blots suggest a single *BEAF* gene. Studying both proteins will be necessary to understand this discrepancy.

### Enhancer Blocking by scs′ Requires Integration into the Genome

The requirement of chromosomal integration for enhancer blocking, combined with the previous demonstration that scs′ works in a directional manner such that it does not inhibit enhancer activity when placed upstream of the enhancer in transgenic flies (Kellum and Schedl, 1992), indicates that scs′ does not directly interfere with enhancer–promoter interactions. DNA transiently transformed into cells may not be properly packaged into chromatin, or may not be compartmentalized in the nucleus to permit enhancer-blocking activity, or both. In contrast, the su(Hw) protein can block enhancer activity in both transient assays and transformed cells in the presence of reiterated binding sites (Holdridge and Dorsett, 1991), suggesting that su(Hw) acts through a different mechanism. We do not observe a specific inhibition by scs′ when the same constructs are tested for the heat shock response. The distance between scs′ and the ecdysone response element is about 400 bp, while this distance is only about 140 bp for the dominant heat shock element. This difference in spacing may permit the formation of a chromatin enhancer block for the former but not the latter enhancer. Of course, other explanations, such as enhancer specificity in the blocking response, are not ruled out and need to be studied.

Other DNA elements have been described that exert their biological activity only when tested by stable transformation. These are LCRs, which confer high level and developmentally regulated expression to the genes of the vertebrate β-globin locus (see, e.g., Grosveld et al., 1987), and scaffold-associated regions (SARs). LCRs were previously mentioned; SARs consistently stimulate expression of heterologous reporter genes in various biological systems after integration into the genome (Mielke et al., 1990; Klehr et al., 1991; reviewed by Laemmli et al., 1992), although they do not confer position-independent gene expression to reporter genes (Poljak et al., 1994). Common to these sequences may be an ability to function over several kilobases of DNA by establishing specific, far-reaching chromatin structures, nuclear compartmentalization, or both. Elucidating the presumptively structural signaling pathway involving chromatin, nuclear organization, or both will be an important direction for future research.

### Implication of CGATA and BEAF-32 in Boundary Function

Our data implicate BEAF-32, the complete BEAF activity, and the CGATA target sequences in boundary function. The enhancer blocking experiments using the 7-mer repeats of the 48 bp BTS and m2-BTS oligonucleotides are consistent with this notion. The reduction in enhancer blocking upon mutating CGATA motifs in the BTS 7-mer and D fragments was observed in two to three different transfections, each of which consisted of pools of several hundred individual stable clones. One might have expected a larger effect upon mutation of the palindrome, although it is possible that other proteins, such as those present in the LM complex, are involved whose interactions are not abolished by the mutations. Furthermore, if chromatin structure is indeed involved, then our constructs were not optimally made. Perhaps an efficient boundary demands at least two palindromes with the correct spacing between them, as in scs′. A comparative study using more optimal, minimal boundary constructs might provide a more sensitive assay for the identification of the relevant features.

How do boundary elements impose their biological functions: serving as barriers against the influx of repressive chromatin and preventing promiscuous enhancer interactions? Our experiments suggest via chromatin/nuclear structure, although they do not eliminate any models. Considerably more detailed structural and biochemical studies are necesssary to approach a mechanistic understanding of this phenomenon. However, these experiments inspire confidence that a clearer understanding of the role of higher order chromatin structure in the regulation of gene expression is forthcoming.

Note: subsequent to the submission of this paper, we isolated a second cDNA clone, which encodes a protein we refer to as BEAF-32B. This protein differs from BEAF-32 (now referred to as BEAF-32A) only in the amino terminus and generates a footprint similar to that generated by the Drosophila BEAF activity.

## Experimental Procedures

### Cells, Embryos, Nuclei, and Nuclear Extracts

The Drosophila tissue culture cell line KC 161 was maintained in D22 medium at 25°C as described (Mirkovitch et al., 1984). Drosophila tissue culture cell line D1 was grown in Schneider's medium (GIBCO) containing 10% fetal bovine serum as described (Dübendorfer, 1986). Wild-type Oregon-R D. melanogaster were reared at 25°C as described (Gasser and Laemmli, 1986). Nuclei were prepared as described (Mirkovitch et al., 1984).

Nuclear extracts were prepared by extracting nuclei on ice for 30 min in 1 ml of 10 mM HEPES (pH 7.6), 350 mM KCl, 0.1 mM EDTA, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.2% Trasylol, 0.2 mM PMSF, 1 μg/ml each of antipain, leupeptin, chymostatin, and pepstatin A per 100 $OD_{260}$ units, followed by centrifugation at 150,000 × g for 60 min. The supernatant was dialyzed into BSK-250 (25 mM HEPES [pH 7.6], 250 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mM PMSF). KC nuclei yielded about 50 μg of protein per $OD_{260}$ unit.

### Plasmids and DNA Methods

The scs′ core was kindly provided by Paul Schedl as an XbaI fragment in pBR322. The scs′ core subfragments, diagrammed in Figure 1B, were ligated into the SmaI site of pBluescript KS (Stratagene). In brief, subfragment A is an XbaI–MspI fragment (positions −5 to 163 relative to the G of the EcoRI site); B is an SspI–HgiAI fragment (positions 101 to 279); C is an MspI–MboII fragment (positions 162 to 352); D is an HgiAI–BamHI fragment (positions 284 to 459); E is an MboII–XbaI fragment (positions 354 to 508). While checking the orientation of insertion and the endpoints of the cloned fragments by dideoxy sequencing, we found one plasmid to contain pBluescript KS sequences from position 2459 to position 2628. This fragment, called BS, was subsequently used as a negative control sequence. Constructs for the enhancer blocking assay were made by inserting different boundary and control elements into the PstI site at −129 in the *hsp27* promoter of a derivative of p1200CAT (Riddihough and Pelham, 1986). pAcneo was constructed by inserting the neomycin resistance gene into pPac (Krasnow et al., 1989). pAcluc was constructed by inserting the actin 5C promoter from pAcneo into pRSV-luc (de Wet et al., 1987). All DNA manipulations were performed by use of standard procedures (Sambrook et al., 1989).

### Gel Mobility Shift Assay

DNA binding reactions typically contained 2 × $10^4$ dpm gel isolated, EcoRI–XbaI cut probe (1 fmol), 5 μg of poly(dI–dC) (less as the BEAF activity was purified), 2 μg of nuclear extract (or 0.5–2.0 μl of column fractions), and gel-isolated specific competitor as indicated, in a volume of 15 μl of BSK-100. After 15 min at 22°C, the reaction mixtures were electrophoresed at 10–15 V/cm on 4% polyacrylamide, 2.5% glycerol gels in 0.25× TBE, dried, and exposed to an X-ray film at −70°C.

### scs′-Binding Protein Purification

KC nuclear extracts were loaded onto PC (Whatman) at 3–4 mg of protein per ml of PC and eluted by a linear gradient of NaCl (0.25 M to 1 M) in BSN buffer (BSK, except NaCl instead of KCl). BEAF activity eluted between 370 mM and 420 mM NaCl. The 44 bp BTS oligonucleotide encoding the BEAF-binding site determined by OP-Cu footprinting (5′-CAAAGTCACGATATTCTTCCACCAACCGATAGTATCGCACACTA, with five GATC overhangs on both strands) was concatenated and coupled to cyanogen bromide–activated Sepharose CL-4B (Pharmacia) at an estimated concentration of 50 μg/ml resin and used to affinity-purify PC fractions containing peak BEAF activity as described by Kadonaga (1991), except that the elution buffer was 25 mM HEPES (pH 7.6), 0.1–1.0 M KCl, 0.1 mM EDTA, 1 mM DTT, 0.1% NP-40, and 20% glycerol. BEAF activity eluted in the 400 mM to 700 mM KCl steps. After the DNA affinity chromatography was repeated, the 500 and 600 mM KCl fractions contained the BEAF activity peak.

### Footprinting

The D subfragment was labeled on one end and gel isolated. Treatment of preparative gel shift assay gels with OP-Cu was done according to the protocols of Sigman et al. (1991); treatment of DNA with DMS and subsequent cleavage by piperidine was done according to those of Wissmann and Hillen (1991); and treatment of DNA with hydroxyl radical was done according to those of Dixon et al. (1991). Preparative gel shift assay reactions were performed such that 50% of 5 × $10^5$ dpm probe was present in the BEAF complex. DNA was recovered from the gels according to the protocols of Sigman et al. (1991). Methylated DNA was then cleaved with piperidine. Samples were electrophoresed on 6% or 8% polyacrylamide, 7 M urea gels in TBE buffer together with G reactions as size standards. Gels were dried and exposed to an X-ray film at −70°C.

### Cloning of *BEAF-32* cDNA

The BEAF doublet (30 μg, from 10 liters of KC culture) was transferred from an SDS–polyacrylamide gel onto an Immobilon polyvinylidene difluoride membrane (PVDF; Millipore), and the excised proteins on the PVDF membrane were individually subjected to tryptic digestion followed by fractionation of the peptides by reverse phase HPLC using a C18 column (2.1 × 250 mm; Vydac) and sequencing of selected peptides, using the gas phase sequencer 470A (Applied Biosystems) as described by Adachi and Laemmli (1992). The amino acid sequence of peptide 1 of the upper protein band (WEEYEQNDEISEDIK), which encompassed peptide 1 of the lower band (EEYEQN), was used to design a degenerate oligonucleotide (5′-GCTCTAGATGGGA(AG)GA(AG)TA(TC)GA(AG)CA, where nucleotides in parentheses represent mixtures).

PCR amplification was done by the RACE protocol described by Frohman et al. (1988). cDNA was synthesized by using 0.5 μg of total RNA from KC cells with 400 ng of oligo(dT) primer (5′-CTAGCGGCCGCTCGAGATCGA(T)17, provided by M. Nishizawa). After ethanol precipitation, 0.1 μg of the cDNA was mixed with 100 pmol each of the general primer (5′-CTAGCGGGCGCTCGAGATCGAT) and the BEAF-specific degenerate primer and subjected to 30 cycles of PCR amplification (94°C, 1 min; 65°C, 2 min; 72°C, 3 min). The amplified band was excised from a 1% agarose gel and reamplified. The reamplified product was cloned into pBluescript KS(−) and sequenced.

The amplified partial cDNA (0.8 kb) was used to screen a plasmid cDNA library from 4–8 hr embryos (Brown and Kafatos, 1988) according to the protocols of Sambrook et al. (1989). One positive clone was isolated from 8 × $10^4$ colonies. The cDNA insert was subcloned into pBluescript KS(−), and both strands were sequenced with a Pharmacia T7 dideoxy sequencing kit.

### Bacterial Expression of BEAF-32

The NsiI–EcoRI fragment of the *BEAF-32* cDNA was cloned into NsiI–EcoRI cut pET3-b(NSEB), a derivative of the pET3-bT7 expression vector in which the original EcoRI site was destroyed by inserting a polylinker, and protein was expressed as described by Studier et al. (1990). The expressed BEAF-32 was purified by PC and DNA affinity column chromatography.

### Antibodies

Chicken antibodies were raised against the upper protein of the purified BEAF doublet isolated after SDS–PAGE, according to the protocols of Adachi and Laemmli (1992). Rabbit antibodies were raised against BEAF-32 protein expressed in Escherichia coli. BEAF-32 (100 μg) eluted from an SDS gel was mixed with complete adjuvant and injected into a rabbit. The rabbit was boosted with 50 μg of the protein after 3 weeks and 5 weeks, and blood was collected from week 5. Both the chicken and the rabbit antibodies were affinity-purified according to the protocols of Adachi and Laemmli (1992).

### Immunostaining of Polytene Chromosomes

Polytene chromosome squashes were prepared from late third instar larvae salivary glands and immunostained according to the protocols of Weeks et al. (1993). As the primary antibody, affinity-purified chicken antibodies were used at 1:2 dilution, and affinity-purified rabbit antibodies were used at 1:20 dilution. A 1:200 dilution of FITC-conjugated rabbit anti-chicken or goat anti-rabbit secondary antibodies was used. The DNA of RNase A–treated squashes was stained with 100 ng/ml propidium iodide. The coverslip was mounted with 6 μl of antifading mix (78% glycerol, 1 mg/ml paraphenylene diamine in PBS) and sealed with nail polish. The chromosome preparations were viewed and photographed through a Bio-Rad MRC 600 confocal or Zeiss Axiophot microscope.

### In Vivo Formaldehyde Protein–DNA Cross-Linking and Analysis after Immunoprecipitation and DNA Amplification

All procedures were essentially as described by Orlando and Paro (1993). The average size of the sonicated chromatin used for isopycnic CsCl centrifugation was 0.5 kb. The CsCl-purified fixed chromatin (30 μg) in 300 μl was immunoprecipitated by adding 10 μl of affinity-purified rabbit anti-BEAF-32 antibody and rotating at 4°C overnight, followed by a 3 hr 4°C incubation with 20 μl of protein A–Sepharose beads. After RNase A and proteinase K treatments and organic extractions and back-extractions, the DNA was precipitated in the presence of 20 μg of glycogen.

The DNA eluted from the protein A–Sepharose beads both with and without antibodies was estimated to be approximately 1 ng and so was amplified by ligation-mediated PCR (Mueller and Wold, 1989). The linker was prepared from two oligonucleotides, a 25-mer of sequence

5′-GCGGTGACCCGGGAGATCTGAATTC and an 11-mer of sequence 5′-GAATTCAGATC. The ligated mixture was amplified by PCR with the 25-mer oligonucleotide described above as primer, and the PCR products were inspected on a 1.5% agarose gel and analyzed by slot blot.

### Transfections

Stable transfections of D1 cells under G418 selection were done according to Rio and Rubin (1985), using 10 μg of linearized CAT vectors and 1 μg of neomycin resistance gene vector (pAcneo) coprecipitated with calcium phosphate. Hundreds of G418-resistant colonies appeared after about 2 weeks. The cells were grown as a mixed culture. Transient transfections were done as the stable transfections except that the transfection efficiency control vector (luciferase gene) pAcluc was substituted for pAcneo and the cells were harvested after 48 hr without G418 selection. Ecdysone and heat shock treatment were done as described by Riddihough and Pelham (1986), CAT extracts were prepared and assayed as described by Sambrook et al. (1989), and luciferase activity was assayed as described by de Wet et al. (1987).

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**GenBank Accession Number**

The accession number for the complete cDNA sequence is X86007.